Deciphering responses of black pepper (*Piper nigrum* L.) to endophytic colonization by *Pseudomonas putida* BP25

Ph.D Final Synopsis

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Ph.D. Final synopsis to the Ph.D work entitled "Deciphering responses of black pepper (*Piper nigrum* L.) to endophytic colonization by *Pseudomonas putida* BP25" is as given below.

1. INTRODUCTION

Black pepper (*Piper nigrum* L.), the 'King of Spices' is an important condiment for India (Ravindran 2000). Crop losses due to diseases and pests are identified as the major causes for low productivity of pepper in India (Sarma et al. 1994; Sarma and Anandaraj 1997). Black pepper is affected by several diseases caused by fungi, bacteria, virus, and phytoplasma, besides nutritional disorders. Among the diseases affecting black pepper, foot rot caused by *Phytophthora capsici* and slow decline caused by *Radopholus similis* are the major diseases (Ravindran 2000). Beyond good agronomic and cultural practices, growers often rely heavily on chemical pesticide application (Daayf et al. 2003). However, the development of resistance to many pesticides in major crop and post-harvest pathogens and public concern over pesticide residues in food and the environment have created interest in alternative methods of disease control (Shahid et al. 2003; Heydari 2007).

Biocontrol of crop diseases has emerged as a promising option (Khetan 2001; Daayf et al. 2003). Beneficial bacteria are known to improve plant health by plant growth promotion

and disease suppression. An alternative mechanism for biological control is that bacterial metabolites affect the plant in such a way as to increase the plant's resistance to pathogens, a process termed induced systemic resistance (ISR). Induced resistance is a physiological "state of enhanced defensive capacity" elicited by specific environmental stimuli, whereby the plant's innate defences are potentiated against subsequent biotic challenges (van Loon et al. 1998). This enhanced state of resistance is effective against a broad range of pathogens and parasites, including fungi, bacteria, viruses, nematodes, parasitic plants and even insect herbivores (Benhamou and Nicole 1999; Kessler and Baldwin 2002; McDowell and Dangl 2000; Sticher et al. 1997; van Loon et al. 1998). The two most clearly defined forms of induced resistance are systemic acquired resistance (SAR) and induced systemic resistance (ISR) which can be differentiated on the basis of the nature of the elicitor and the regulatory pathways involved. SAR can be triggered by exposing the plant to virulent, avirulent, and non-pathogenic microbes or artificially with chemicals (Sticher et al. 1997). Both ISR and SAR represent a state of enhanced resistance of the plant that depends on the signalling compounds jasmonic acid and ethylene (Knoester et al. 1999; Yan et al. 2002) and salicylic acid (Cameron et al. 1994; Uknes et al. 1992; Ward et al. 1991), respectively. The expression of rhizobacteria induced ISR can involve different mechanisms such as growth promotion (developmental), reduced symptom expression (physiological) and induction of cell wall reinforcement, phytoalexins and pathogenesis related proteins (biochemical) as well as an enhanced capacity to express these defence responses up on challenge inoculation with a pathogen, a mechanism known as priming (Conrath et al. 2006).

Pseudomonas sp. has been used extensively as biological control agents against many soil borne plant pathogens in several crops (Weller 1988). Various mechanisms are involved in disease suppression by soil pseudomonads, viz. production of siderophores, volatile organic compounds (VOCs), HCN and antibiotics, competition, induction of systemic resistance etc. (Weller and Thomashow 1993). Introduced biocontrol bacteria that can reside within the plant system as endophytes provide added advantage. Endophytic bacteria are defined as "bacteria that live within living plant tissue without doing substantial harm or gaining benefit other than securing residency" (Hallmann et al. 1997). Among the diverse group of endophytic bacteria, *Pseudomonas* is recognized as a useful microbial candidate for plant disease management. The intimate relationship between the endophytic bacteria and their hosts make them a natural choice for selection as biocontrol agents (Van Buren et al. 1993; Chen et al. 1995).

Pseudomonas putida, a gram negative and rod shaped soil saprophyte, has been called a beneficial organism because it can enhance plant yield and suppress certain root fungal pathogens (Kloepper et al. 1980). The aggressive colonization of root surfaces by *P. putida* may be involved in these beneficial phenomena as well as being an important survival strategy for the bacterium. Interactions between plants and beneficial bacteria can have a profound effect on crop health, yield and soil quality **(**Kloepper et al.1989; Sturz et al. 2000). Understanding the mechanisms of biocontrol of plant diseases is critical to the eventual improvement and effective use of biocontrol agents. Research on the molecular basis of the endophyte induced resistance is required to understand the plant responses to endophytic colonization and pathogen attack of endophyte inoculated plants.

In our study, *Pseudomonas putida* BP25, an endophyte isolated from black pepper root was used. It was identified as an effective antagonist for biological control of *Phytophthora* foot rot in black pepper (Aravind et al. 2009b). With this background the present investigation is proposed to study the mechanisms by which black pepper plant health is potentiated through endophytic colonization by *P. putida* BP25.

2. OBJECTIVES

The present study will be undertaken with the following specific objectives:

1. To characterize the nature of endophytic colonization of black pepper by *Pseudomonas putida* BP25.
2. To study the plant growth promotion and disease suppression properties of the strain *P. putida* BP25.
3. To decipher the mechanisms of defence related plant responses in black pepper colonized with *P. putida* BP25.

3. REVIEW OF LITERATURE

3.1. Black pepper

Black pepper (*Piper nigrum* L.) known as the "king of spices" is the most widely used spice in the world. It is a perennial climbing vine belonging to the genus *Piper* and family Piperaceae. India is one of the major producer, consumer and exporter of black pepper in the world. Vietnam, Indonesia, Brazil, Sri Lanka, Malaysia and Philippines are the other black pepper growing countries. Black pepper of commerce is the mature dried berry of the pepper plant, which has got a characteristic pungency, aroma and flavour. Black pepper is cultivated to a large extent in Kerala, Karnataka and Tamil Nadu and to a limited extent in Maharashtra, North Eastern states and Andaman & Nicobar Islands. Kerala and Karnataka account for a major portion of the production of black pepper in the country (Ravindran 2000).

Over seventy five cultivars of black pepper are being cultivated in India, Kerala being the richest state in cultivar diversity. Karimunda is the most popular of all cultivars in Kerala. Eighteen improved varieties have been released for cultivation. Panniyur 1 to 8 are the varieties released at the Pepper Research Station, Panniyur (Kerala Agricultural University). Subhakara, Sreekara, Panchami, Pournami, PLD2, IISR-Shakthi, IISR-Thevam, IISR-Girimunda and IISR-Malabar Excel are the varieties released from ICAR-IISR, Kozhikode, Kerala; and Arka Coorg Excel released jointly by ICAR-Indian Institute of Horticulture Research, CHES, Chettali and ICAR-IISR, Regional Station, Appangala, Karnataka (Ravindran et al. 2000, Devasahayam et al. 2015).

3.2. Diseases of black pepper

Black pepper is affected by several diseases besides insect pests and nutritional disorders. One of the important reasons for the decline in black pepper productivity in India is diseases caused by oomycetes, fungi, nematodes, virus and phytoplasma. Among them *Phytophthora* foot rot and slow decline are considered as major diseases of black pepper. *Phytophthora* foot rot, caused by the soil borne oomycete *Phytophthora capsici* is the most devastating among the diseases affecting black pepper. All parts of black pepper including roots, stems and leaves are vulnerable to infection by *P. capsici* resulting in huge economic damage (Sarma and Anandaraj 1997). *Phytophthora* infections are broadly classified into aerial and soil infections. Soil infections cause root rot and collar rot leading to rapid wilting and death of vines. Aerial infection occurs on the runner shoots, leaves, spikes and stems. The infection on leaves appear as dark spots with fimbriate margins which spread rapidly throughout the leaves resulting in defoliation. Infections on the stem are seen as black lesions

resulting in blight (Anandaraj and Sarma 1997). Slow decline (slow wilt) disease is caused by the plant parasitic nematodes - *Radopholus similis*, the burrowing nematode and *Meloidogyne incognita*, the root knot nematode either independently or in combination with *P. capsici* (Ramana and Eapen 1995; Anandaraj et al. 1996a, 1996b). The affected plants survive for years and death of plants occurs gradually over a period of 3–4 years. The affected vines show varying degrees of feeder root loss resulting in aerial symptoms such as foliar yellowing, defoliation, loss of vigour and productivity, leading to slow death of the plant (Ramana 1991). The burrowing nematode, *R. similis* is an important plant parasitic nematode of black pepper. The nematode invades succulent root tips and colonize inter and intracellular cortical cells producing large necrotic lesions throughout the length of the root. Due to severe infestation, the roots lose their function and will not be able to absorb moisture and nutrients from the soil resulting in slow wilt (Mohandas and Ramana 1987). Other diseases of black pepper are anthracnose caused by the fungus *Colletotrichum gloeosporioides*, leaf rot and blight caused by *Rhizoctonia solani*, basal wilt caused by *Sclerotium rolfsii*, stunted disease of black pepper caused by viruses, *Piper yellow mottle virus* (PYMoV) and *Cucumber mosaic virus* (CMV) (Anandaraj and Sarma 1995, Lockhart et al. 1997; Bhat et al. 2003).

Various methods for management of plant diseases involve cultural practices, crop rotation, host resistance, chemical control and biological control. Chemical control measures including spraying and drenching with Bordeaux mixture (1%) and drenching copper oxychloride (0.2%) have been found effective for disease management in black pepper (Ramachandran et al. 1991). Metalaxyl was found to be the most effective chemical among the systemic fungicides tested against *P. capsici* (Ramachandran and Sarma 1985). Chemical pesticides such as phorate and carbofuran are highly effective in reducing *R. similis* populations (Ramana and Eapen 1999). Currently, biological control is emerging as an alternative option for plant disease control (Khetan 2001; Daayf et al. 2003). There are several biocontrol agents to manage diseases in black pepper. Antagonistic microorganisms including fluorescent *Pseudomonas* and *Trichoderma* spp. and Vesicular Arbuscular Mycorrhiza (VAM) have shown promising results in the control of foot rot and slow decline diseases of black pepper (Anandaraj and Sarma 1995; Anandaraj et al. 1991; Anith et al. 2003; Eapen 2008; Diby et al. 2005; Diby and Sarma 2005; Aravind et al. 2009a,b, 2010)

3.3. Endophyte

Plant growth-promoting bacteria (PGPB) are associated with many, if not all, plant species and are commonly present in many environments. The most widely studied group of PGPB are plant growth-promoting rhizobacteria (PGPR) colonizing the root surfaces and the

closely adhering soil interface, the rhizosphere. Some of these PGPR can also enter root interior and establish endophytic populations (Kloepper 1999; Gray and Smith 2005). Endophytic bacteria can be defined as those bacteria that colonize the internal tissue of the plant without any external sign of infection or negative effect on their host (Schulz and Boyle 2006). It is very interesting that among the nearly 3,00,000 plant species that exist on the earth, each individual plant is host to one or more endophytes (Strobel et al. 2004). Many of them are able to transcend the endodermis barrier, crossing from the root cortex to the vascular system, and subsequently thrive as endophytes in stem, leaves, tubers, and other organs (Bell et al. 1995; Compant et al. 2005a; Gray and Smith 2005; Hallmann et al. 1997). The extent of endophytic colonization of host plant organs and tissues reflects the ability of bacteria to selectively adapt to these specific ecological niches. Consequently, intimate associations between bacteria and host plants can be formed without harming the plant (Kloepper et al. 1999). Abundant and diverse populations of bacterial endophytes have been identified in many plants including potato (Sturz et al. 1999); maize (McInroy and Kloepper 1995a), cotton (McInroy and Kloepper 1995b), cucumber (Mahafee and Kloepper 1997) and black pepper (Aravind et al. 2009b). Bacteria isolated from the internal plant tissue of healthy plants comprise over 144 species representing over 55 genera (Kobayashi and Palumbo 2000). The most common taxa of endophytic bacteria recovered from internal plant tissue belong to the genera *Pseudomonas*, *Bacillus*, *Enterobacter*, *Agrobacterium* and *Burkholderia* (Gardner et al. 1982; McInroy and Kloepper 1995a,b; Jacobs et al. 1985; Hallmann et al. 1997; Mahaffee and Kloepper 1997, Mundt and Hinkle 1976; Sturz 1995).

Several *Pseudomonas* spp. were isolated from plant parts of different crop species such as cotton (Mundt and Hinkle 1976), Grape (Bell et al. 1995), Sugarbeet (Jacobs et al. 1985), soybean (Kuklinsky-Sobral et al. 2004), black pepper (Aravind et al. 2009b) etc. Among the various species of the genera *Pseudomonas*, *Pseudomonas putida* is found throughout various environments. *P. putida* were known to show a diverse spectrum of metabolic activities, which is indicative of their adaptation to various niches, which includes the ability to live in soils and sediments contaminated with high concentrations of heavy metals and organic contaminants (Wu et al. 2011). *Pseudomonas putida* strains were also found as rhizospheric and endophytic bacteria and, were identified as beneficial bacteria with proven effect on plant growth and biological control of several plant pathogens (Kloepper et al. 1988). *Pseudomonas putida* BP25, isolated as an endophyte from root tissues of black pepper cultivar Panniyur 5, exhibited antagonistic activity against foot rot causing oomycete, *P. capsici* and a nematode pest of black pepper, *R. similis* (Aravind et al. 2009a,b, 2010).

3.4. Beneficial effects by endophytes

The beneficial effects of bacterial endophytes on their host plant appear to occur through similar mechanisms described for plant growth promoting rhizobacteria (PGPR) (Kloepper et al. 1991; Hoflich et al. 1994). This makes sense because most of the bacterial endophytes isolated from trees, healthy crops, and weeds can be considered to be facultatively endophytic and are capable of living outside plant tissues as rhizospheric bacteria (Di Fiore and Del Gallo 1995). Plant growth promoting rhizobacteria (PGPR) typically promote plant growth in two ways: direct stimulation and biocontrol (i.e. suppressive activity against soil-borne diseases). The positive effect of many soil bacteria on plants is mediated by a range of mechanisms including improvement of mineral nutrition, enhancement of plant tolerance to biotic and abiotic stresses, modification of root development, as well as suppression of soil-borne diseases. The bacterial traits that have been reported to directly facilitate the proliferation of their plant hosts includes nitrogen fixation, synthesis of siderophores that can solubilize and sequester iron from the soil, synthesis of phytohormones such as indole-3-acetic acid (IAA) that can act to enhance various stages of plant growth, mechanisms for the solubilization of minerals such as phosphorus that will become more available for plant growth; and synthesis of some less well-characterized, low-molecular-mass compounds or enzymes that can modulate plant growth and development (Brown 1974; Kloepper et al. 1980, 1989; Lambert and Joos 1989; Glick et al. 1994; Glick 1995; Vessey 2003).

3.5. Mechanisms of biocontrol activity

Several endophytic bacteria have showed promise in control of fungal plant pathogens (Pleben et al. 1995; Sturz and Matheson 1996; Aravind et al. 2009b), insects (Azevedo et al. 2000) and nematodes (Aravind et al. 2009a, 2010). The widely recognized mechanisms of biocontrol mediated by PGPB are competition for an ecological niche or a substrate, production of inhibitory allelochemicals including iron-chelating siderophores, antibiotics, biocidal volatiles, lytic enzymes, and detoxification enzymes, and induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens (Bloemberg and Lugtenberg 2001) and/or abiotic stresses (Mayak et al. 2004).

3.5.1 Endophytic colonization

The rhizosphere competence of biocontrol agents is a prerequisite of effective biological control. It comprises effective root colonization combined with the ability to survive and proliferate along growing plant roots over a considerable time period, in the presence of the indigenous microflora (Parke 1991; Weller 1988). Endophytic bacteria are

known to colonize and establish inside the plant parts. The endophytic niche provides protection to the bacteria and helps these bacteria in functioning efficiently as biocontrol agents. There are various suitable nutrient-rich niches along root surfaces attracting a great diversity of microorganisms. Competition for these nutrients and niches is a fundamental mechanism by which PGPB protects plants from phytopathogens (Duffy 2001). The endophytic bacteria are reported to colonize intercellular spaces of the root epidermal cells and vascular tissue, but also have been isolated from all plant parts including seeds (Chen et al. 1995; Hallmann et al. 1997; M'Piga et al. 1997).The main entry for endophytic bacteria appears to be through wounds that occur naturally as a result of plant growth or through root hairs and at epidermal junctions (Sprent and de Faria 1988). Active penetration of endophytic bacteria in the absence of external wounding in seedlings suggests the involvement of cellulose production by them (Quadt-Hallmann et al. 1997). The cell wall degrading enzymes endogluconase and polygalacturonase seem to be required for the infection of *Vitis vinifera* by *Burkholderia* sp. (Compant et al. 2005b). Although the root zone offers the most obvious site of entry for many endophytes, entry may also occur at sites on aerial portions of plants such as flowers (Sharrock et al. 1991), stomata (Roos and Hattingh 1983) or through stem lenticels (Kluepfel 1993). Once inside the plant tissue, endophytic bacteria remain localized in a specific plant tissue, such as the root cortex, or colonize the plant systematically by transport or active migration through the conducting elements or the apoplast (Hurek et al. 1994; Quadt-Hallmann et al. 1997). Endophytic populations, like rhizospheric populations, are conditioned by biotic and abiotic factors (Fuentes Ramirez et al. 1999; Hallmann et al. 1997; Seghers et al. 2004). The highest bacterial densities are usually observed in the roots and decrease progressively from the stem to the leaves (Lamb et al. 1996).

Studies on bacterial endophytes have focused on characterization of isolates obtained from plant tissues following disinfection with sodium hypochlorite or similar agents (Miche and Balandreau 2001). For visualization of colonization of bacteria, green fluorescent protein (GFP) technique has been employed effectively and it has facilitated the detection *Pseudomonas* BCAs on the roots of crop plants (Bloemberg et al. 2000), *Pseudomonas* sp. ph6 in ryegrass (*Lolium multiflorum* Lam.) (Sun et al. 2014) and *P. fluorescens* PICF7 in olive roots (Prieto et al. 2011). Colonization of poplar trees (Germaine et al. 2004), pea plants (Germaine et al. 2006) and olive roots by *P. putida* have also been demonstrated by GFP technique. Recently, quantitative real time PCR (q-PCR) was developed as an invaluable tool for estimating the population levels of endophytes. Quantification of *Herbaspirillum*

seropedicae strain SmR1, an endophytic diazotroph in maize roots (Pereira et al. 2014) and *Gluconacetobacter diazotrophicus* in sugarcane by real time PCR (Sorte et al. 2014) was reported.

3.5.2 Direct antagonism

Endophytic bacteria are known to reduce or prevent the deleterious effects of certain pathogenic organisms. Diseases caused by fungi, bacteria, nematodes, virus, and in some instances even damage caused by insects can be reduced following prior inoculation with endophytes (Kerry 2000; Sturz et al. 2000; Ping and Boland 2004; Berg and Hallmann 2006). The disease suppressing ability of *P. putida* has been reported in several crops. *P. putida* was found effective in suppressing *Fusarium* wilt in radish caused by *Fusarium oxysporum* f. sp. *raphani*; *Fusarium* wilt of muskmelon caused by *F. oxysporum* f. sp. *melonis* (Bora et al. 2004), vascular wilt of cotton caused by *F. oxysporum* f. sp. *vasinfectum* (Chen et al. 1995) and tomato foot and root rot caused by *F. oxysporum* f. sp. *radicis- lycopersici* (Forl) (Validov et al. 2007). Biocontrol activity of *P. putida* against several other pathogens such as *Sclerotinia sclerotiorum* in sunflower (Expert and Digat 1995); *Pythium* in sugar beet and in cucumber (Shah-Smith and Burns 1996, Ongena et al. 2000); *Botrytis cinerea* in bean and tomato (Ongena et al. 2004; Adam et al. 2008); and *Rhizoctonia solani* in rice (Krishnamurthy and Gnanamanickam 1998) have also been demonstrated. *P. putida* Ld4 was also identified as a biological control agent for the control of Colorado Potato Beetle (CPB), *Leptinotarsa decemlinea*, a pre-eminent pest on solanaceous crops worldwide (Muratoglu 2011).

It is reported that the metabolites including siderophores, volatile organic compounds (VOCs) lytic enzymes and antibiotics are involved in pathogen suppression (Defago and Haas 1990; Keel et al. 1990; Ryu et al. 2003; Loper and Buyer 1991). Siderophores are low-molecular-weight compounds which competitively acquire ferric ion and are produced under iron-limiting conditions by PGPB (Whipps 2001). The secreted siderophore molecules bind most of the Fe^{3+} that is available in the rhizosphere and as a result effectively prevent any pathogens in the immediate vicinity from proliferating because of a lack of iron (O'Sullivan and O'Gara 1992; Buysens et al. 1994). A bacterial mutant impaired in the production of the fluorescent siderophore pseudobactin, no longer suppressed disease revealing the importance of siderophores in disease suppression (Raaijmakers et al. 1995). Siderophore mediated antagonism was reported to be the mechanism of disease suppression of fluorescent pseudomonads against *P. capsici* in black pepper (Diby et al. 2005). Another way that a PGPR can be employed to prevent proliferation of phytopathogens is through the synthesis of

antibiotics. A variety of antibiotics have been identified, including compounds such as amphisin, 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide, oomycin A, phenazine, pyoluteorin, pyrrolnitrin, tensin, tropolone, and cyclic lipopeptides produced by pseudomonads (Defago 1993; Nielsen and Sorensen 2003; Diby and Sarma 2006). *P. putida* strain BK8661, which produces siderophore, antibiotics, and low levels of hydrogen cyanide was found to suppress growth of *Septoria tritici* and *Puccinia recondite* f.sp.*triticiin vitro* and on wheat leaves (Flaishman et al. 1996). Other important mechanisms include production of lytic enzymes such as chitinases and β-1,3-glucanases which degrade chitin and glucan present in the cell wall of fungi (Lim et al. 1991), HCN production (Defago et al. 1990) and degradation of toxin produced by pathogen (Borowitz et al. 1992; Duffy and Defago 1997).

Volatile organic compounds produced by microorganisms (MVOCs) have been evaluated as a new approach for disease control. MVOCs are defined as compounds having high enough vapour pressures under normal conditions to significantly vaporize and enter the atmosphere (Chaparro et al. 2014). MVOCs belong to various chemical classes, e.g. low molecular weight fatty acids and their derivatives (hydrocarbons, alcohols, aldehydes and ketones), terpenoids, aromatic compounds, nitrogen containing compounds and volatile sulphur compounds (Schulz and Dickschat 2007, Effmert et al. 2012). So far, mostly *Bacillus, Pseudomonas* and *Streptomyces* species have been reported to efficiently inhibit the growth of phytopathogenic fungi through volatile compounds (Zheng et al. 2013; Athukorala et al. 2010; Fernando et al. 2005; Wan et al. 2008; Kai et al. 2007). Bacterial volatiles were shown to inhibit several fungi and oomycetes responsible for major crop diseases including species from the genera *Alternaria, Botrytis, Colletotrichum, Fusarium, Phytophthora, Pythium, Rhizoctonia, Sclerotinia* and *Verticillium* (Weisskopf 2013). Recently, Popova et al. (2014) demonstrated the killing effect of VOCs produced by *Pseudomonas* and *Serratia* strains on cyanobacteria, *Drosophila melanogaster* and *Caenorhabditis elegans*. Nematicidal activities of bacterial volatiles were also reported against *Panagrellus redivivus, Bursaphelenchus xylophilus* and *Meloidogyne incognita* (Gu et al. 2007; Huang et al. 2010). Gas chromatography-Mass spectrometry (GC-MS) has been widely utilized for chemical profiling of volatile compounds emitted from bacteria (Chuankun et al. 2004; Farag et al. 2006; Tenorio-salgado et al. 2013).

3.5.3. Induced systemic resistance (ISR)

Induced resistance is defined as an enhancement of the plant's defensive capacity that is effective against a broad spectrum of pathogens and pests and is acquired after appropriate stimulation. This induced disease resistance is referred to as either systemic acquired

resistance (SAR) when the inducer is a necrotizing pathogen (Hammerschmidt and Kuc 1995) or induced systemic resistance (ISR) when the inducing agent is a nonpathogenic rhizobacterium (Van Loon et al. 1998). Upon infection with a challenging pathogen this enhanced defensive capacity is manifested as a reduction in the rate of disease development, resulting in fewer diseased plants or in lesser disease severity. SAR is expressed to a maximum level when the inducing organism causes necrosis (Cameron et al. 1994) whereas ISR by PGPR typically do not cause any necrotic symptoms on the host plants (Van Loon et al. 1998). The inducing rhizobacteria triggers a reaction in plant roots that spread systemically throughout the plant and thus extends the protective action of PGPR from their antagonistic activity against soil-borne pathogens in the rhizosphere to a defense-stimulating effect in aboveground tissues against foliar pathogens (Leeman et al. 1995).

PGPR induce resistance in plants against fungal, bacterial and viral diseases (Liu et al. 1995a, b; Maurhofer et al. 1998), insect (Zehnder et al. 1997) and nematode pests (Oostendorp and Sikora 1989, 1990). ISR has been established as a mechanism that is effective in bean, carnation, cucumber, radish, tobacco, tomato, as well as in the model plant *Arabidopsis thaliana*, against different types of pathogens (Van Loon et al. 1998; Ramamoorthy et al. 2001). PGPR that elicit ISR in one plant species may not do so in another, indicating specificity in the interaction between rhizobacteria and plants (Bakker et al. 2003; Van Loon and Bakker 2005). Fluorescent *Pseudomonas* spp. was reported to be the major group of rhizobacteria that trigger ISR. *Pseudomonas* spp. such as *Pseudomonas fluorescens* G8-4, *P. putida* 34-13 and *P. aureofaciens* elicited systemic protection against cucumber anthracnose (Wei et al. 1991) and *Pseudomonas denitrificans* strain 1-15 in oak trees against *Ceratocystis fagacearum* (Brooks et al. 1994). *P. fluorescens* strain Pf1 induced resistance against different pathogens in different crops, viz., *Rhizoctonia solani* (Nandakumar 1998), *Colletotrichum falcatum* in sugarcane (Viswanathan 1999) and *Pythium aphanidermatum* in tomato (Ramamoorthy et al. 1999) indicating the broad spectrum activity of PGPR mediated ISR.

PGPR can also induce systemic protection against bacterial and viral diseases. *P. fluorescens* strain 97 protected beans against halo blight disease caused by *Pseudomonas syringae* pv. *phaseolicola* (Alstrom 1991), while treatment of cucumber seed with *P. putida* strain 89B-27 decreased the incidence of bacterial wilt disease (Kloepper et al. 1993) and angular leaf spot caused by *Pseudomonas syringae* pv. *lachrymans* (Liu et al. 1995b). Induction of systemic resistance by PGPR against viral diseases has been reported in cucumber, tomato and tobacco plants. *P. fluorescens* strain 89B-27 has consistently reduced

the number of cucumber mosaic virus-infected plants (CMV) and delayed the development of symptoms in cucumber and tomato (Raupach et al. 1996). In tobacco, soil-application of *P. fluorescens* strain CHAO has induced systemic protection against inoculation with tobacco necrosis virus (TNV) (Maurhofer et al. 1994, 1998). The role of ISR in insect pest management has also been demonstrated. Induction of resistance by *Pseudomonas gladioli* against *Helicoverpa armigera* in cotton plants (Qingwen et al. 1998) and systemic resistance by *P. putida* strain 89B-27 against *Acalyma* v*ittatum* and *Diabrotica undecimpunctata howardi* in cucumber were reported (Zehnder et al. 1997). Induced systemic resistance against nematode pest is reported in sugar beet against *Heterodera schachtii*, the cyst nematode (Oostendorp and Sikora 1989, 1990).

Mechanism of PGPR mediated ISR

PGPR bring about ISR through reinforcing the physical and mechanical strength of the plant cell wall as well as changing the physiological and biochemical reaction of the host leading to the synthesis of defence chemicals against the challenge pathogen. Cell wall fortification is the first line of defence mechanism against the spread of a pathogen (Benhamou et al. 1996b, M'Piga et al. 1997). Cell modifications include lignifications, formation of structural barriers, viz., cell wall apposition (papillae) and deposition of newly formed callose and accumulation of phenolic compounds (He et al. 2002). *P. fluorescens* strain 63-28 has resulted in formation of structural barriers, viz., cell wall apposition (papillae) and deposition of newly formed callose and accumulation of phenolic compounds against *Pythium ultimum* and *F. oxysporum* f. sp. *pisi* (Benhamou et al. 1996a, M'Piga et al. 1997). Lignification of cell wall was reported in bean by *P. putida* (Anderson and Guerra 1985). Phenylalanine ammonia-lyase (PAL) which has been related to lignification and synthesis of defense related phenolics was induced in the early stages of interaction between bean roots and bacterial endophytes (Zdor and Anderson 1992).

Increased levels of peroxidase (PO), catalase and polyphenol oxidase (PPO) along with PAL were detected in leaves apart from the roots of rhizobacteria treated plants indicating the systemic protection offered to black pepper against foliar infection by the pathogen, *P. capsici* (Diby and Sarma 2005). Systemic protection conferred by *P. putida* was associated with accumulation of PO in bean (Albert and Anderson 1987), phytoalexins in cucumber (Ongena et al. 2000) and lipoxygenase in tomato (Adam et al. 2008). In many cases ISR by PGPR is associated with the accumulation of PR proteins (pathogenesis-related proteins) such as chitinases and β-1,3 glucanases (Maurhofer et al. 1994; Benhamou et al.1996a; M'Piga et al. 1997), synthesis of phytoalexin and other secondary metabolites (Zdor

and Anderson 1992). Papaya fruits treated with *P. putida* MGP1 showed a significant lower disease index against post-harvest blight caused by *Phytophthora nicotianae* and demonstrated increases in chitinase, β-1,3-glucanase, PAL, PO, PPO, catalase activities and total phenolic content (Ying et al. 2013). But, *P. fluorescens* strain WCS417r has induced protection in radish and *Arabidopsis* without activating PR gene expression indicating the possibility of other defence compounds in ISR (Van Wees et al. 1997; Pieterse et al. 1996).

Salicylic acid (SA), jasmonate (JA) and ethylene were emerged as important signalling molecules in plant defense (Pieterse et al. 1996, Van Wees et al. 1997). Resistance induced by some strains of *Pseudomonas* sp. was associated with increased levels of SA (Maurhofer et al. 1994; Zhang et al. 2002). In contrast, *P. fluorescens* induced ISR in *Arabidopsis thaliana*, was shown to be ethylene and JA dependent but SA independent (Ton et al. 2002; Knoester et al. 1999; Iavicoli et al. 2003; Pieterse et al. 1996; Van Wees et al.1997). The existence of cross-talk between these signalling pathways has also been proven which help the plant to either prioritize the action of a particular pathway over another or activate multiple pathways if necessary (Dong 1998; Reymond and Farmer 1998; Pieterse and van Loon 1999).

The development of molecular-biological techniques has allowed the reaction of plants to rhizobacteria to be determined at the transcriptional level by analyzing differential gene expression by a variety of techniques. Few studies have been published describing the molecular basis of the interactions between endophytic bacteria and plants. Microarray analysis has been employed in studying the differential expression of *A. thaliana* upon inoculation with *P. putida* strains (Srivastava et al. 2012; Sheoran et al. 2016) and *P. fluorescens* (Wang et al. 2005). Verhagen et al. (2004) reported local responses exerted in *Arabidopsis* roots challenged with *P. fluorescens* WCS417r using an *Arabidopsis* GeneChip microarray. A new and highly effective method, termed suppression subtractive hybridization (SSH), a PCR based technique developed by Diatchenko et al. (1996) has also been used for investigating differential gene expression by generating subtracted cDNA library. The technique has already been used in various plant species to isolate transcripts involved in responses to biotic and abiotic stresses (Birch et al. 1999; Beyer et al. 2001; Montesano et al. 2001; Kang et al. 2003). SSH followed by qRT-PCR confirmed induction of various defence related transcripts in *P. fluorescens* PICF7 colonized olive roots and aerial tissues (Schiliro et al. 2012; Gomez-Lama Cabanas et al. 2014), *P. chlororaphis* O6 colonized cucumber (Kim et al. 2004) and *P. fluorescens* C7R12 colonized *Medicago truncatula* (Sanchez et al. 2005).

4. MATERIALS AND METHODS

4.1. Endophytic colonization

Bacterial strain and growth conditions

The strain *P. putida* BP25 was initially isolated from black pepper root (Aravind et al. 2009b). The gfp tagged rifamycin resistant strain of *P. putida* BP25 obtained from IARI, New Delhi was used to track endophytic colonization in black pepper (*Piper nigrum* L. cv. Sreekara). BP25R::gfp was cultured on Luria Bertani agar (LBA, Himedia) amended with rifampicin (50 µg/ml) and gentamicin (75 µg/ml) at 37°C for 24h. Overnight cultures of BP25R::gfp in LB adjusted to 1.0 O.D (absorbance at 600 nm) were used for plant inoculation.

Plant species and growth conditions

The roots of two-leaf stage black pepper cuttings (*Piper nigrum* L. cv. Sreekara) were treated with bacterial suspension (10^8 cfu/ml) for 30, 60 and 120 min, respectively. The treated rooted cuttings were planted in pots containing 500 g of potting mixture (sterile coir pith: farmyard manure 3:1) and grown under greenhouse conditions at 28-30°C with 60-80% humidity. Sampling was done at weekly intervals (7^{th}, 14^{th}, 21^{st} and 28^{th} days). The endophytic population was confirmed by dilution plating, Bio-PCR and fluorescent microscopy.

Dilution plating

The bacterized black pepper plants (30, 60 & 120 min) were aseptically partitioned into roots, shoots and leaves. One gram of tissue was surface sterilized with 0.5% sodium hypochlorite + 0.01% tween 20 for 20 min , 70% ethyl alcohol for 1 min followed by rinsing thoroughly with sterile distilled water for 2-3 times. Each sample was ground aseptically with 2 ml of PBS (PBS, gL^{-1} NaCl 8, KCl 0.2, Na_2HPO_4 1.44, KH_2PO_4 0.24, pH 7.4) and serially diluted up to 10^{-3}. One ml of serially diluted samples was pour plated in LBA amended with rifampicin (50 µg ml^{-1}) and gentamicin (75 µg ml^{-1}) and incubated at 28°C for 48 h. Colonies were counted and expressed as CFU per gram of fresh tissue.

Bio-PCR

Bio-PCR was adopted to confirm the endogenous population in plant. It involves culturing an organism prior to PCR amplification, making it a particularly useful method for increasing the bacterial numbers to detectable levels in asymptomatic tissue and ensuring that only viable organisms are examined. Plant extracts were prepared as in the case of plate count after surface sterilization. Then, 1 ml of the extract was inoculated into 9 ml LBB

amended with rifampicin (50 µg ml^{-1}), gentamicin (75 µg ml^{-1}) and incubated at 28°C for 48 h. After incubation, 2 µl of broth culture was used as template for PCR using primer tpiA_F: 5´-CGAATTCGTCTGCGTTCAG-3´; tpiA_R: 5´-GAGCTGACCAAAGGCTTGAG-3´ that is known to yield 667 bp amplicon.

Development of a quantitative Real time PCR method for detecting endogenous population

Real time primers 16 S SS1_FP: 5' CAACGTTTCGAAAGGAACG 3' and 16S SS1_RP: 5' GTCGCCTTGGTGAGCCATT 3' were designed from the 16S rRNA gene of *P.putida* to yield 131 bp amplicon. The specificity of the primers was tested by using genomic DNA from other bacteria and the sensitivity of the assay was analysed by running different dilutions of BP25R DNA ranging from 100 ng to 0.1 pg. Real time PCR was carried out in Qiagen Rotor-Gene Q. For constructing a standard curve, different dilutions of BP25R DNA ranging from 26 ng to 1 pg were run. A standard curve was constructed by plotting Ct values relative to the corresponding concentration of template DNA.

For quantifying endogenous population in black pepper, rooted cuttings grown in sterile soilrite were dipped in BP25R cell suspension (O.D- 1.0) and planted. In control treatments, cuttings were treated with distilled water. Sampling was done after 7, 14, 21 and 28 days post inoculation. Plants were partitioned into roots, shoots and leaves and surface sterilized as described earlier. Plant part (100 mg) was ground in CTAB extraction buffer using a sterile mortar and pestle and total genomic DNA was extracted (Ausubel et al. 2002). Genomic DNA from three plants per interval were pooled and used to assess colonization. Real time PCR reactions were carried out in 20 µl of PCR mixture containing 10 µl SYBR green master mix (HotStar Taq Polymerase, SYBR green I, SYBR Green PCR buffer and reference dye), 1 µl of 10 µM forward and reverse primers and 1 µl template using the same thermocycling conditions mentioned above. In negative controls, genomic DNA was replaced by water to rule out contamination. Ct values from reactions containing bacterial DNA alone were correlated with the amount of DNA and the Ct values obtained for wells containing DNA from plant parts were interpolated in calibration curves to calculate the quantity of the bacterial DNA with respect to the total.

Fluorescence microscopy

Colonization pattern of *P. putida* BP25 in black pepper plantlets were visualized using fluorescence microscopy. Sections were made from roots colonized with BP25 and

mounted in distilled water under a coverslip. Fluorescent microscopic observations were performed using Leica 5000B microscope and images were photographed.

4.2. Plant growth promotion and disease suppression properties of *P. putida* BP25

4.2.1 Plant growth promotion

P. putida BP25 was screened for various plant growth promoting properties such as siderophore production and IAA (indole 3-acetic acid) production. Production of siderophores was checked on chrome azurol S (CAS) agar containing chrome azurol S and hexadecyltrimethylammonium bromide (HDTMA) as indicators. For screening for IAA production, bacteria were inoculated into nutrient broth supplemented with 0.2% of L-tryptophan and incubated for 10 days at 28°C. Detection of IAA in supernatant was done using Salkowski reagent and quantification was done spectrophotometrically by reading O.D at 530 nm.

4.2.2 Disease suppression through direct antagonism

Bioassay for antagonistic activity of P. putida BP25 on plant pathogens

Dual plate confrontation assays were performed to test the activity of bacterial volatiles on the growth and development of pathogens. BP25 was tested against a range of pathogens *P. capsici*, *P. myriotylum*, *A. rolfsii*, *R. solani*, *G. moniliformis* and *C. gloeosporioides*. Mycelial disk from three-day-old potato dextrose agar (PDA)/carrot agar (CA) cultures of fungal and oomycetes pathogen were placed in the middle of PDA/CA in Petri dish. Similarly 200µl of 10^9 cfu/ml mid-log phase bacterial culture was spread on TSA (HiMedia, India) in another Petri plate. The lids of both the plates were removed and the inoculated plates were positioned face to face and fastened using parafilm in such a way that the pathogen was directly exposed to bacterial volatiles released during the growth of bacteria. The plates were incubated at a common optimum temperature of 28°C for 3-5 days and observed for mycelial growth inhibition. The plates without bacterial inoculum served as mock. BP25 was also tested against the plant parasitic nematode, *R. similis*. Dual plate confrontation assays were performed by sealing the TSA plate with BP25 culture against water agar (1.5%) containing approximately 25 nematodes. The plates were incubated at 26°C for 3 days and mortality of *R. similis* was calculated.

Antimicrobial activity of synthetic volatile compounds in vitro

The MVOCs released from *P. putida* BP25 were profiled using dynamic head space Gas Chromatography Mass Spectrometry and compounds such as 2,5-dimethyl pyrazine, 2-methyl pyrazine, 2-ethyl 5-methyl pyrazine, 2-ethyl 3,6-dimethyl pyrazine and dimethyl

trisulphide were identified. Chemically synthesized volatile compounds as identified in head space GC/MS analysis of BP25 were procured from Sigma Aldrich USA and used for the study. MVOCs were evaluated against various pathogens such as *P. capsici*, *P. myriotylum, R. solani*, *G. moniliformis*, *A. rolfsii*, *C. gloeosporioides* and *R. similis*. For fungal bioassay, PDA was inoculated with mycelial disc and sealed against a lidless plate containing filter paper impregnated with volatile compound. The amount taken was adjusted so as to get different concentrations such as 21 µg/cm^3, 42 µg/cm^3, 84 µg/cm^3, 169 µg/cm^3, 339 µg/cm^3, 509 µg/cm^3 and 679 µg/cm^3. After incubation, mycelial diameter was measured and % inhibition was calculated. For *R. similis*, water agar (1.5%) was used instead of PDA and live/dead nematodes were counted after incubation and % mortality was calculated.

In planta assays for MVOCs on black pepper cut shoots against P.capsici

Healthy cut shoots of black pepper with approximate length of 8-10 cm were used in this experiment. Cut shoots were surface sterilized with 0.1% sodium hypochlorite for 1 minute, rinsed with sterile distilled water 3-4 times and air dried. Surface sterilized cut shoots were then inoculated with 3 mm *P. capsici* mycelial discs at one end. To study the activity of MVOCs, the inoculated cut shoots were arranged in a plastic box and exposed to the compounds. The volume of the box was calculated and accordingly different concentrations such as 21 µg/cm^3, 42 µg/cm^3, 84 µg/cm^3, 169 µg/cm^3, 339 µg/cm^3, 509 µg/cm^3 and 679 µg/cm^3 of the compounds were added on to cotton balls placed in the box with no direct contact with the pathogen. Moistened cotton was kept inside the box to ensure moisture. The boxes were then sealed tightly to prevent the escape of volatiles and kept for incubation for 3 days at 26°C under greenhouse conditions.Cut shoots inoculated with *P. capsici* alone served as control. Cut shoots exposed to chemicals alone were also kept to check toxicity of compounds. After incubation, cut shoots were observed for lesion suppression/toxicity and lesion length was measured. % inhibition was calculated using the formula,

% inhibition = (lesion length in control-lesion length in treated)×100/lesion length in control

MVOCs as a soil fumigation agent against fungal pathogens and R.similis

For fumigation assays, approximately 200 g of sterile potting mixture taken in a 2 L container was inoculated with 5 mycelial discs each of *P. capsici*, *P. myriotylum, R. solani*, *G. moniliformis*, *A. rolfsii* and *C.gloeosporioides.* The amount of the compounds was calculated so as to get different concentrations such as 100 µg/cm^3, 50 µg/cm^3, 25 µg/cm^3, 12.5 µg/cm^3, 6.25 µg/cm^3, 3.125 µg/cm^3 and 1.5625 µg/cm^3 and added on to cotton balls placed in the container with no direct contact with the pathogen. The container is then sealed

tightly to prevent the escape of the MVOCs and incubated at 26°C for 5 days. After incubation, mycelial discs were transferred on to Rose Bengal agar for checking viability. For *R. similis*, potting mixture was inoculated with approximately 200 *R. similis* and was then exposed to MVOCs in a tightly sealed container. After 48 h, potting mixture was sieved by Cobb's sieving method and total nematodes were isolated from the concentrated sample by centrifugal floatation method using sucrose (specific gravity 1.18). Total numbers of live and dead nematodes were counted and % mortality was calculated.

4.3. Plant responses in black pepper to *P. putida* BP25 colonization

4.3.1. Biochemical analysis

Extraction and estimation of phenols

Extraction and estimation of phenols was done by Folin-Ciocalteau assay. Absorbance of the samples was measured at 725 nm in a spectrophotometer and quantified against a standard of gallic acid. The phenolic content was reported as gallic acid equivalents based on a standard curve.

Determination of lignin content

Lignin was determined by acetyl bromide procedure (Liyama et al. 1990). Absorbance was measured at 280 nm and % of lignin content was calculated using SAC (Specific Absorption Coefficient) of lignin, 20 $g^{-1}cm^{-1}$.

Estimation of defence related enzymes

Defence related enzymes such as phenylalanine ammonia lyase (PAL), peroxidase (POX) and polyphenol oxidase (PPO) were estimated by enzyme activity assays. Rooted cuttings were treated with BP25 and planted. Sampling was done at 24 h, 48 h, 3 d, 4 d, 5 d, 7 d, 10 d and 14 d. Root and leaf extracts were analysed for defence enzymes.

Estimation of peroxidase (PO) activity

Quantitative estimation of peroxidase was done according to the method described by Jennings et al. (1969). Change in absorbance was read at 470 nm for 3 min. The enzyme activity was expressed as change in absorbance per min per mg of protein.

Estimation of polyphenol oxidase (PPO) activity

Polyphenol oxidase activity was measured as described by Mayer et al. (1965) using catechol. The change in absorbance was read at 495 nm for 3 min and the enzyme activity was expressed as changes in absorbance per min per mg of protein.

Estimation of phenylalanine ammonia lyase (PAL) activity

Determination of PAL was done following the method of Peltonen and Karjalainen (1995). Reaction mixture containing 2.75 ml of 0.2% L- phenylalanine in 50 mM Tris-HCl (pH 8.5) and 0.25 ml of enzyme extract was incubated for 1 h at 37°C after which the O.D was recorded at 290 nm.

Estimation of catalase activity

Determination of catalase activity was done following the method of Dekock et al. 1960. The absorbance was read at 240 nm at every 15 sec interval and the time required for decrease in absorbance by 0.05 units was recorded.

4.3.2. Molecular responses

RNA extraction and mRNA purification

In order to get a wide range of induced transcripts, bacterized and non-bacterized roots were sampled at different times such as 24 h, 48 h, 3 day, 5 day and 7 day (two plants/time point). Roots were flash frozen, ground in liquid nitrogen and total RNA was extracted from treated and control samples for each time point using TRI reagent (Sigma, USA). RNA samples corresponding to each treatment (BP25 inoculated and control plants) were pooled separately to obtain two independent RNA pools. Poly A+RNA was purified from 400 µg of total RNA using mRNA Purification Kit (MN, Germany) following manufacturer's instructions. The quality of mRNA was checked on 1% agarose gels and its quantity as well as purity was checked spectrophotometrically using a biophotometer.

Generation of a suppression subtractive hybridization (SSH) cDNA library

The SSH library enriched with differentially expressed genes was generated using the PCR-Select cDNA subtraction kit (Clontech Laboratories, USA) following the manufacturer's instructions. Firstly, cDNAs were separately synthesized from 1 µg of mRNA of each BP25 inoculated (tester) and control (driver) plants. The cDNAs were purified, dissolved in nuclease free water and then digested with *Rsa1* to obtain short and blunt ended fragments. *RsaI* digested tester cDNA was purified, divided in to two subpopulations, a and b; and was ligated to adaptor 1 and adaptor 2R at 16°C overnight. Each adaptor ligated tester population was then hybridized separately with an excess of driver cDNA and incubated at 98°C for 1.5 min and then at 68°C for 8 h. After incubation, the reactions were mixed together for a second subtractive hybridization with driver cDNA at 68°C overnight. After incubation, 30 µl dilution buffer was to the subtracted cDNA and incubated at 68°C for 7 min. Two rounds of PCR amplification were performed to enrich differentially expressed

sequences. Primary PCR amplification was carried out in 25 µl final volume using PCR primer 1 provided in the kit. Secondary PCR was carried out using nested PCR primers provided in the kit. Enriched subtracted cDNAs obtained were ligated in the pTZ57R/T (Thermo Scientific, USA) and cloned into *Escherichia coli* DH5α cells using InsTAclone PCR Cloning Kit (Thermo Scientific, USA). Positive transformants were selected based on white/blue colour selection on LB agar with ampicillin, X-gal and IPTG and confirmed by colony PCR using M13 primers. Plasmids of one hundred bacterial clones of the resulting SSH library were eventually isolated and sequencing was done using M13 universal primer (Scigenom Labs Pvt. Ltd., Cochin).

Bioinformatics analysis of expressed sequences tags (ESTs)

The sequences of the clones were checked for vector contamination using VecScreen and trimmed from vector and adaptors sequences. Low quality and short (<100 bp) sequences were excluded from the analysis. Computational annotation of ESTs from the black pepper-PpBP25R interaction was performed using 'Blast2GO version 3.3.5. Homologies were checked in the NCBI protein database by running blastx with the E-value set to $1.0e^{-1}$. Blast2GO software was used to obtain Gene Ontology (GO) information from retrieved database matches and annotation of all sequences was performed. InterProScan analysis was applied to find functional motifs by using the specific tool in the Blast2GO software with the default parameters. Enzyme mapping of annotated sequences was retrieved by direct GO to enzyme annotation and used to query the Kyoto Encyclopaedia of Genes and Genomes (KEGG) define the main metabolic pathways involved.

Data validation and expression profile analysis by quantitative real-time PCR

Quantitative Real-Time PCR (qRT-PCR) experiments were carried out to validate the induction of selected ESTs presumed to have functional roles. Five ESTs such as pathogenesis related protein (PR-4), catalase, glutathione S-transferase, metallothionein and WRKY transcription factor 40 were selected for q-RT PCR validation. Specific primer pairs for each sequence were designed using the primerQuest tool. Total RNA was isolated from roots (both treated and control) sampled at different time points; 24 h, 48 h, 3 d, 5 d and 7 days after post inoculation. cDNA was synthesized from 2 µg of DNase treated total RNA using reverse transcriptase (Fermentas). qRT-PCR experiments were carried out using QuantiFast SYBR Green mastermix (Qiagen, Germany). Melting curves of qRT-PCR products were assessed from 62°C to 99°C to confirm the amplification of single PCR products. qRT-PCR analyses were finally performed using Rotor gene Q Real Time PCR System (Qiagen, Germany). Relative fold differences were calculated based on the

comparative Ct method using actin as an internal standard. To determine relative fold differences for each sample in each experiment, the Ct values for the genes were normalized to the Ct value for the reference gene actin and were calculated using the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001).

5. RESULTS

5.1. Endophytic colonization of *P. putida* BP25 in black pepper

In black pepper, BP25 showed a spatiotemporal pattern in endophytic colonization. The bacterium colonized the rhizoplane and root in high population but not the stem and leaf by dilution plating. Results of the colony counts indicated stable population densities up to 5-6 $\log_{10}$ CFU per gram in rhizoplane and up to 4 $\log_{10}$ CFU in roots (Fig. 1). Duration of bacterization did not affect the bacterial colonization as the endogenous population size for 30 min, 60 min and 120 min was nearly same. However, highly sensitive Bio-PCR using BP25R::gfp specific primer yielded a specific amplicon of 667 bp in stem that further confirmed the presence of bacteria at a lower level (Fig. 2). A specific and rapid q-RT PCR method was developed for the quantification of BP25R in black pepper. No non specific amplification could be detected with endophytes other than PpBP25R confirming the specificity of the primers. Melting curve analysis confirmed the specific amplification product. A standard curve was constructed by plotting Ct values relative to the corresponding concentration of template DNA. Ct values obtained for tubes containing DNA from plant parts were interpolated in calibration curves to calculate the quantity of the bacterial DNA with respect to the total. DNA concentration quantified by q-PCR was 0.4 ng/µl during 7^{th} and 14^{th} days and 3.7 pg/µl and 4.6 pg/µl during 21^{st} and 28^{th} days post inoculation respectively (Fig. 3). BP25R could be visualized on the root surface and in the intercellular spaces of treated plants under fluorescence microscope.

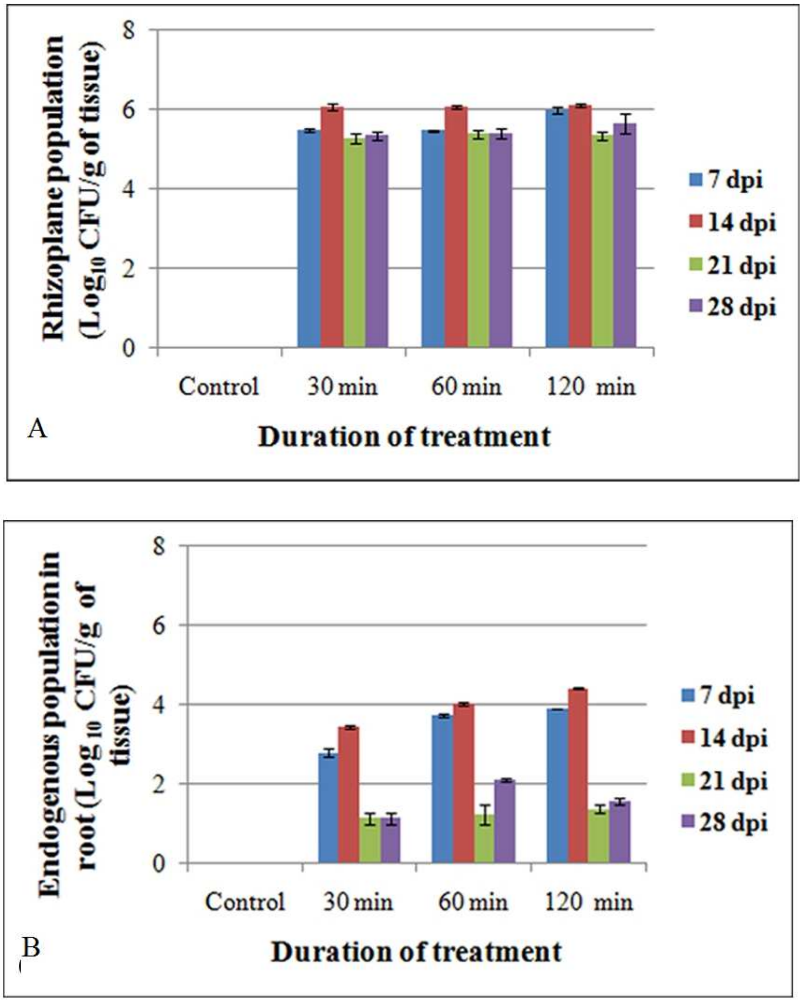

Fig. 1 Colonization pattern of *Pseudomonas putida* BP25 in black pepper A. Rhizoplane B. Root

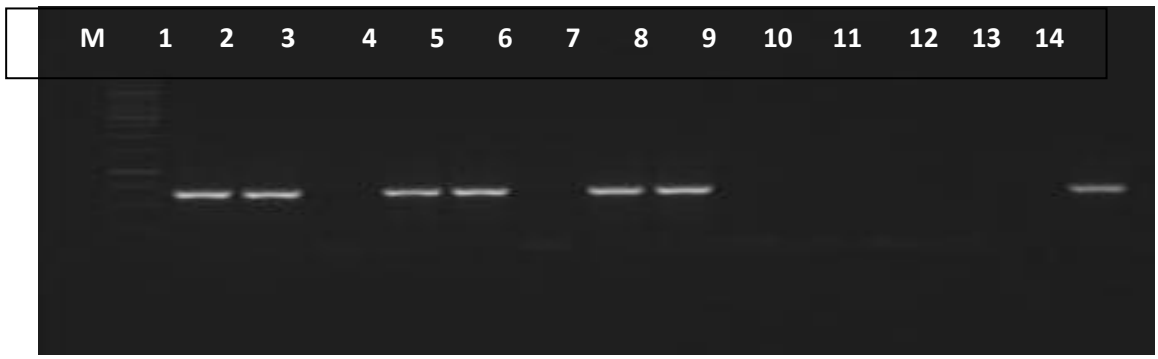

Fig. 2 Bio-PCR based detection of BP25 R::gfp in black pepper: Lane M- 1Kb ladder; Lane 1, 2, 3 Root, stem & leaf of plantlet treated for 30 min; Lane 4, 5, 6 - Root, stem & leaf of plantlet treated for 60 min; Lane 7, 8, 9 - Root, stem & leaf of plantlet treated for 120 min; Lane 10, 11, 12 -Root, stem & leaf of control; Lane 13- Negative control (No template DNA); Lane 14- Positive control.

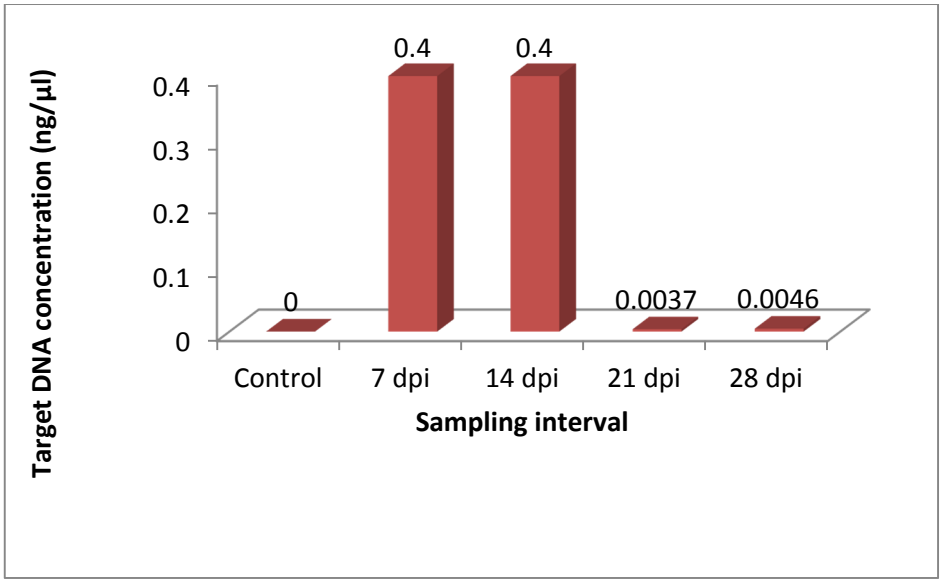

Fig. 3 Quantification of *Pseudomonas putida* BP25 in black pepper roots by real time PCR

5.2. Plant growth promotion and disease suppression properties

5.2.1. Plant growth promotion

Potential growth promoting traits such as siderophores and IAA production were observed with BP25. Development of yellowish orange halo on chrome azurol S (CAS) agar indicated the production of siderophores by *P. putida* BP25. BP25 also gave red colour in tryptophan containing medium on incubation with Salkowski reagent indicating positive result for IAA and the concentration was quantified to be 16 µg/ml.

Disease suppression through direct antagonism

Bioassay for antagonistic activity of P. putida BP25 on plant pathogens

The volatiles released during the growth of BP25 were found inhibitory to a broad range of pathogens *P. capsici*, *A. rolfsii, R. solani, G. moniliformis C. gloeosporioides* and *R. similis*. All the oomycetal and fungal pathogens were inhibited to the level of 62.2% to 85.9% on TSA. Inhibitory activity was also confirmed on a synthetic medium, M9 minimal medium which yielded inhibition level ranged from 80% to 90% (Table 1, Fig. 4). The % mortality for the volatiles against *R. similis* was 92%.

Table 1 Activity of volatiles on TSA and M9 minimal media against different pathogens

Pathogen	% inhibition	
	TSA	M9 Minimal medium
Phytophthora capsici	81.8	90.0
Rhizoctonia solani	74.4	80.0

Athelia rolfsii	85.9	87.7
Gibberella moniliformis	62.2	89.3
Colletotrichum gloeosporioides	85.9	86.6
Radopholus similis	92.0	92.0
C.D	3.22	7.40
SE (m)	0.95	2.18
SE(d)	1.34	3.08

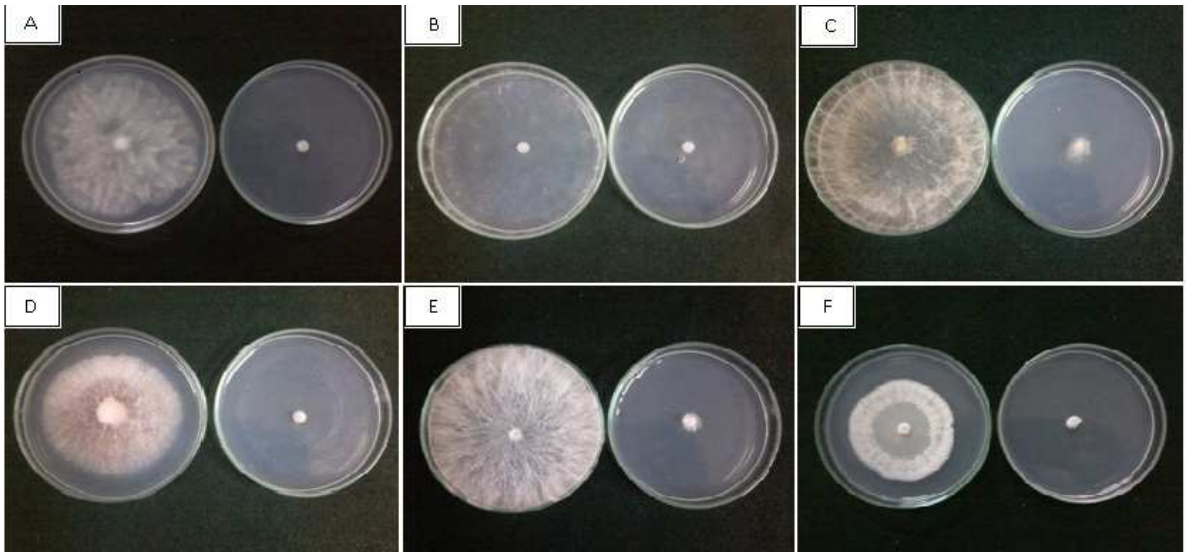

Fig. 4 Antagonistic activity of BP25R volatiles on M9 minimal agar against different pathogens A: *P. capsici* control and exposed, B: *P. myriotylum* control and exposed, C: *R. solani* control and exposed, D: *G. moniliformis* control and exposed, E: *A. rolfsii* control and exposed, F: *C. gloeosporioides* control and exposed.

Antimicrobial activity of synthetic volatile compounds in vitro

Chemically synthesized volatile compounds such as 2,5-dimethyl pyrazine, 2- methyl pyrazine, 2-ethyl-5-methyl pyrazine, 2-ethyl 3,6-dimethyl pyrazine and dimethyl trisulfide showed significant inhibitory activity against all the fungal pathogens. Among the compounds, dimethyl trisulfide showed 100% inhibitory activity at all the tested concentrations against all the pathogens. So it was further tested at lower concentrations and the minimum concentration showing 100% activity against all the pathogens was found to be 2.65 µg/cm^3. EC 50, EC 90 and EC 95 were calculated using probit analysis (Table 2). 2-ethyl 3,6-dimethyl pyrazine was found to be the most effective pyrazine compound against majority of pathogens. Among the pathogens, *R. solani* was the most sensitive to pyrazine compounds. Among the compounds, dimethyl trisulfide was found to have strong nematicidal activity. It exhibited 100% mortality against *R. similis* even at a low concentration of

2.65µg/cm^3. Pyrazine compounds showed 32% to 58% mortality at a concentration of 679 µg/cm^3 (Table 3).

Table 2 EC 50, EC 90 and EC 95 values of volatile compounds produced by *Pseudomonas putida* BP25 against various oomycete and fungal pathogens

Pathogen	Effective concentration	Volatile compounds identified in *Pseudomonas putida* BP25			
		2,5-dimethyl pyrazine	2-methyl pyrazine	2-ethyl 5-methyl pyrazine	2-ethyl 3,6-dimethyl pyrazine
Phytophthora capsici	EC 50	70.2	175.4	103.6	66.1
	EC 90	410.4	900.7	331.4	244.8
	EC 95	748.1	1571.0	491.9	382.1
Athelia rolfsii	EC 50	105.9	129.8	44.5	34.0
	EC 90	285.6	395.9	107.6	67.3
	EC 95	400.2	578.3	145.3	85.0
Colletotrichum gloeosporioides	EC 50	84.3	138.0	73.7	50.3
	EC 90	351.5	370.9	173.5	89.0
	EC 95	571.0	519.2	232.2	108
Gibberella moniliformis	EC 50	95.7	114.0	55.0	44.3
	EC 90	575.0	827.3	190.4	103.0
	EC 95	1058	1624.0	290.4	137.3
Rhizoctonia solani	EC 50	71.9	51.7	41.0	13.0
	EC 90	165.2	146.0	64.0	35.3
	EC 95	219.3	207.8	75.3	49.5

EC 50, EC 90 and EC 95 values for positive controls

Pathogen	Effective concentration	Metalaxyl	Pathogen	Effective concentration	Carbendazim	Pathogen	Effective concentration	Carbendazim
Phytophthora capsici	EC 50	0.37	*Gibberella moniliformis*	EC 50	0.84	*Rhizoctonia solani*	EC 50	0.67
	EC 90	2.17		EC 90	2.42		EC 90	1.09
	EC 95	3.96		EC 95	3.47		EC 95	1.29

*The recommended chemicals, metalaxyl and carbendazim were used for comparison

Table 3 Nematicidal activities of MVOCs against *Radopholus similis*

MVOCs	Mortality (%) at different concentrations (µg/cm^3)			CD
	84	339	679	
2, 5-dimethyl pyrazine	0.0 (0.0)	26.5 (30.94)	50.0 (44.98)	5.664
2-methyl pyrazine	0.0 (0.0)	5.25 (12.24)	31.7 (34.23)	11.06
2-ethyl 5-methyl pyrazine	0.0 (0.0)	29.75 (33.0)	55.0 (47.86)	7.118
2-ethyl 3,6-dimethyl pyrazine	9.0 (16.37)	39.0 (38.62)	57.75 (49.47)	15.03
Dimethyl trisulfide	100.0 (90.0)	100.0 (90.0)	100.0 (90.0)	NS

In planta assays for MVOCs on black pepper cut shoots against P. capsici

Among the tested compounds, the minimum concentration of 2,5 dimethyl pyrazine, 2-ethyl 5-methyl pyrazine and 2-ethyl 3,6-dimethyl pyrazine showing lesion suppression with no signs of toxicity was 21 µg/cm^3 and that of 2-methyl pyrazine was 42 µg/cm^3 (Table 4). Dimethyl trisulfide was found to be effective at lower concentrations, showing 30%, 36% and 41% lesion suppression at 1.32, 2.65 and 5.3 µg/cm^3 respectively.

Table 4 Effect of pyrazine compounds at different concentrations on lesion caused by *Phytophthora capsici* on black pepper cut shoots

Volatile organic compound	Inhibition (%) at different concentrations (µg/cm^3)							
	10.6	21	42	84	169	CD $p< 0.05$	SE (m)	SE (d)
2-methyl pyrazine	30.5 (33.5)	63.0 (52.4)	100.0 (90.0)	100.0 (90.0)	100.0 (90.0)	3.69	1.19	1.68
2, 5-dimethyl pyrazine	49.0 (44.4)	100.0 (90.0)	100.0 (90.0)	100.0 (90.0)	*phytotoxic	3.81	1.19	1.69
2-ethyl 5-methyl pyrazine	55.0 (47.9)	100.0 (90.0)	100.0 (90.0)	100.0 (90.0)	*phytotoxic	0.69	0.22	0.31

2-ethyl 3,6-dimethyl pyrazine	78.0 (62.1)	100.0 (90.0)	100.0 (90.0)	100.0 (90.0)	*phytotoxic	2.02	0.63	0.89
Compound (µg/cm^3)	1.32	2.65	5.3	10.6	21	CD p< 0.05	SE (m)	SE (d)
Dimethyl trisulfide	30.0(33.1)	36.0(36.9)	41.0(3 9.9)	*Phyt otoxic	*Phytotoxic	NS	2.26	3.19

MVOCs as a soil fumigation agent against fungal pathogens and R. similis

Dimethyl trisulfide showed 100% inhibition against *P. capsici*, *R. solani* and *A. rolfsii* at a concentration of 6.25 µg/cm^3 and against *C. gloeosporioides* and *G. moniliformis* at 12.5 µg/cm^3. Dimethyl trisulfide was also seen as an excellent fumigant against *R. similis* (Table 5). No live nematodes could be isolated from the soil samples fumigated at concentrations 100 µg/cm^3 and 50 µg/cm^3 showing 100% mortality. Lower concentrations such as 25 µg/cm^3, 12.5 µg/cm^3, 6.25 µg/cm^3, 3.125 µg/cm^3 and 1.5625 µg/cm^3 exhibited 68%, 53%, 38%, 19% and 5% mortality respectively.

Table 5 Effect of soil fumigation with dimethyl trisulfide on *Radopholus similis*

Concentration of dimethyl trisulfide (µg/cm^3)	% mortality
100	100.0 (90.0)
50	100.0 (90.0)
25	68.0 (55.6)
12.5	53.0 (46.7)
6.25	38.0 (37.9)
3.125	19.0 (25.7)
1.5625	5.0 (12.9)
C.D (p< 0.05)	9.36
SE (m)	2.75
SE (d)	3.89

5.3. Plant responses in black pepper to *P. putida* BP25 colonization

5.3.1. Biochemical responses

Total phenols

A significant increase in total phenols was observed in both root and leaf of bacterized plants. It was around 0.2 to 1.1 fold high compared to control. The highest increase, 1.1 fold was observed on 4th day followed by 1.0 fold on 6th day in roots (Fig. 5a and b).

Lignin content in roots

The lignin content in roots and leaves was higher in bacterized plants compared to control. The highest increase in roots was observed on 6th day after inoculation (Fig. 5c and d).

Defence related enzymes

Bacterized plants showed higher peroxidase activity compared to control. Maximum increase was observed on 4th day, 5th day and 7th day in roots with 1.36, 1.15 and 1.2 fold respectively. In leaves, highest activity was observed on 2nd day and 3rd day with 1.0 fold increase. Polyphenol oxidase activity in bacterized roots was at par compared to control though comparatively higher activity was recorded on 4th day. But in leaves, a slight increase could be observed on 3rd day. A significant increase in phenyl alanine ammonia lyase (PAL) was observed in both roots and leaves of bacterized plants. The highest increase in roots was on 5th day (1.7 fold) followed by 4th day (1.4 fold) and 3rd day (1.0 fold). In leaves, highest activity was on 7th day (1.1 fold). Catalase activity was high in inoculated plants compared to control. The highest increase in roots was recorded on 7th day (2.8 fold) followed by 4th day (1.6 fold). In leaves, comparatively higher activity was observed on 2nd and 7th day after bacterization.

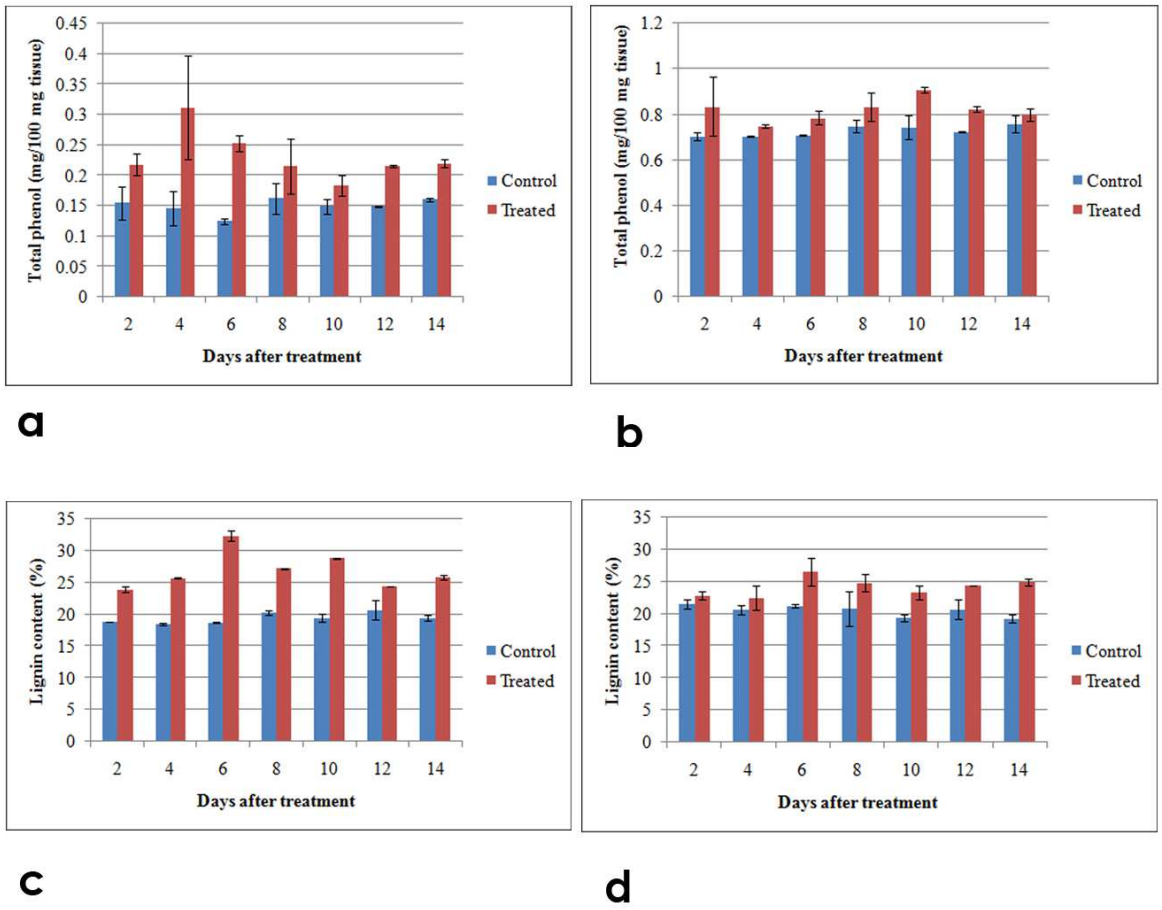

Fig. 5 Total phenol content in root (a) and leaf (b) and; lignin content in root (c) and leaf (d) of uninoculated (control) and *Pseudomonas putida* BP25 treated black pepper plants at different time intervals; 2, 4, 6, 8, 10, 12 and 14 days after treatment. An independent sample t test showed that the difference in quiz scores between the control group and the experimental group were statistically significant; t values are 5.04 ($p < 0.001$) and 4.45 ($p < 0.001$) for root (a) and leaf (b); and 6.56 ($p < 0.001$) and 5.84 ($p < 0.001$) for root (c) and leaf (d).

5.3.2. Molecular responses

Generation of cDNA library by SSH

A forward subtracted cDNA library was generated by SSH to obtain a no. of ESTs, representing genes up-regulated in black pepper upon endophytic colonization by *P. putida* BP25. Insert size of the clones were determined using colony PCR (Fig. 6). A total of 100 ESTs were sequenced. Low quality and short length (>100 bp) sequences were excluded from analysis. Sequence length varied from 154-621 bp with an average length of 426 bp. Querying Blastx allowed hits for 60 sequences (Table 6).

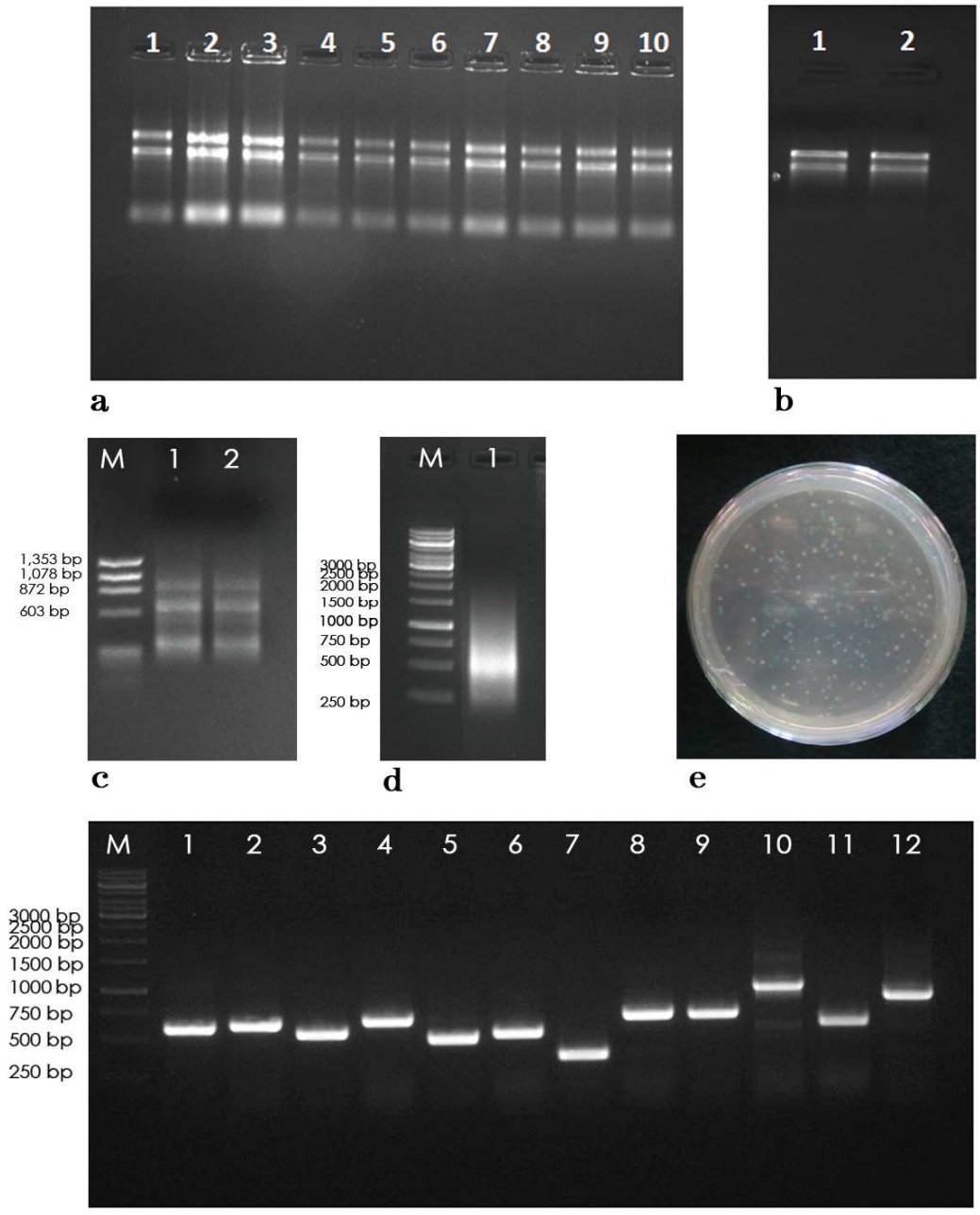

Fig. 6 Construction of a subtracted cDNA library by SSH. a) Total RNA isolated from black pepper root samples inoculated with endophytic *Pseudomonas putida* BP25 (Lane 1-5) and mock inoculated control (Lane 6-10); b) Poly A+ RNA isolated from treated total RNA pool (Lane 1) and control (Lane 2); c) Secondary PCR products: PCR control subtracted cDNA provided in the kit (Lane 1), control subtracted cDNA (Lane 2) and ϕX174/Hae III Digest DNA marker (Lane M); d) Secondary PCR product of experimental subtracted cDNA (Lane 1) and 1 Kb ladder (Lane M); e) Petriplate showing transformed colonies of subtracted cDNA; f) Colony PCR of recombinant clones using vector specific M13 primers for different insert sizes (Lane 1- 12) and 1 Kb ladder (Lane M).

Table 6 *Pseudomonas putida* BP25R induced EST sequences in black pepper roots identified after Blast2GO analysis

Sequence Name	Sequence Description	Sequence Length	Blast E-Value Min	Blast Top Hit Species
BPC1	Pleiotropic drug resistance 7	376	1.40E-42	*Elaeis guineensis*
BPC11	Type 2 metallothionein	424	3.70E-16	*Ilex paraguariensis*
BPC24	WRKY transcription factor 40	475	1.40E-11	*Coffea canephora*
BPC27	Major pollen allergen Bet v 1-D H	458	2.80E-41	*Populus trichocarpa*
BPC33	26S protease regulatory subunit 7-like	431	1.80E-22	*Populus trichocarpa*
BPC36	Type 2 metallothionein	423	3.40E-17	*Camellia sinensis*
BPC46	Pathogenesis-related 1-like	621	1.18E-50	*Nelumbo nucifera*
BPC49	Elongation factor 1-alpha	479	3.50E-82	*Cicer arietinum*
BPC51	Heat shock protein 70	555	3.00E-84	*Ziziphus jujube*
BPC60	Glutathione S-transferase F13-like	385	1.00E-21	*Nelumbo nucifera*
BPC61	Type 2 metallothionein	427	4.20E-17	*Ilex paraguariensis*
BPC65	Type 2 metallothionein	423	3.40E-17	*Camellia sinensis*
BPC69	Catalase partial	476	7.10E-68	*Manihot esculenta*
BPC72	Pathogenesis-related PR-4-like	335	7.80E-20	*Brassica rapa*
BPC77	High mobility group B 1-like	506	1.10E-42	*Nelumbo nucifera*
BPC79	Metallothionein type 2	413	6.30E-18	*Pyrus x bretschneideri*
BPC80	Heat shock protein 70	459	7.00E-91	*Ziziphus jujube*
BPC85	Major pollen allergen Bet v 1-D H	558	9.50E-52	*Nelumbo nucifera*
BPC157	Pathogenesis-related PR-4-like	438	1.00E-55	*Brassica rapa*
BPC166	Major pollen allergen Bet v 1-D H	494	2.00E-46	*Populus trichocarpa*
BPC171	Glutathione S-transferase F13-like	391	1.90E-20	*Nelumbo nucifera*
BPC179	Cell number regulator 8	532	7.90E-59	*Prunus avium*
BPC191	Uncharacterized mitochondrial protein	292	6.30E-44	*Nicotiana tabacum*
BPC220	Major pollen allergen Bet v 1-D H	598	6.40E-50	*Nelumbo nucifera*
BPC224	Ubiquitin	342	7.80E-26	*Nicotiana benthamiana*
BPC229	Major pollen allergen Bet v 1-D H	564	8.70E-52	*Nelumbo nucifera*
BPC244	uncharacterized protein	514	3.30E-02	*Sesamum indicum*
BPC258	5-enolpyruvylshikimate-3-phosphate synthase	526	2.40E-100	*Elaeis guineensis*
BPC269	Type 2 metallothionein	456	4.80E-17	*Camellia sinensis*
BPC273	Pathogenesis-related 1-like	295	3.50E-16	*Populus trichocarpa*
BPC283	Type 2 metallothionein	425	4.20E-17	*Ilex paraguariensis*

BPC285	Major pollen allergen Bet v 1-D H	448	1.60E-37	*Nelumbo nucifera*
BPC298	Inositol oxygenase	296	9.90E-55	*Nelumbo nucifera*
BPC301	Histone	365	4.88E-32	*Glycine soja*
BPC302	Pathogenesis-related PR-4-like	415	3.30E-49	*Brassica rapa*
BPC304	Major pollen allergen Bet v 1-D H	588	1.20E-51	*Nelumbo nucifera*
BPC308	High mobility group B 1-like	529	4.20E-43	*Nelumbo nucifera*
BPC310	Heat shock protein 70	378	1.70E-38	*Eriobotrya japonica*
BPC314	Glutathione S-transferase F13-like	384	1.90E-15	*Nelumbo nucifera*
BPC316	Major pollen allergen Bet v 1-D H	519	2.70E-48	*Populus trichocarpa*
BPC321	Catalase partial	509	3.90E-67	*Ananas comosus*
BPC323	dnaJ homolog	393	5.60E-33	*Gossypium raimondii*
BPC324	Elongation factor 1-alpha	375	5.70E-51	*Cicer arietinum*
BPC326	Major pollen allergen Bet v 1-D H	477	1.60E-41	*Populus trichocarpa*
BPC328	Type 2 metallothionein	440	4.10E-17	*Camellia sinensis*
BPC335	Major pollen allergen Bet v 1-D H	458	2.80E-41	*Populus trichocarpa*
BPC337	Plasma membrane intrinsic protein 1,4 isoform 1	425	1.40E-66	*Pyrus x bretschneideri*
BPC338	NADPH--cytochrome P450 reductase-like	484	2.90E-36	*Oryza sativa Indica Group*
BPC341	Proline transporter 2	477	2.20E-21	*Musa acuminata subsp. malaccensis*
BPC344	Pathogenesis-related 1-like	276	8.80E-17	*Populus trichocarpa*
BPC348	Actin-depolymerizing factor 7-like	304	1.50E-32	*Setaria italica*
BPC353	Elongation factor 1-alpha	476	2.40E-81	*Cicer arietinum*
BPC354	Superoxide dismutase [Cu-Zn]	542	2.60E-94	*Phoenix dactylifera*
BPC358	60S ribosomal L5	587	6.10E-100	*Musa acuminata subsp. malaccensis*
BPC368	Hypothetical protein	518	1.40E-48	*Morus notabilis*
BPC370	Casein kinase II subunit alpha like	394	5.94E-49	*Coffea canephora*
BPC372	Elongation factor 1-alpha	505	3.20E-79	*Cicer arietinum*
BPC373	Metallothionein type 2	478	1.10E-16	*Pyrus x bretschneideri*
BPC387	Type 2 metallothionein	475	6.70E-17	*Camellia sinensis*
BPC389	dnaJ homolog	424	3.20E-32	*Gossypium raimondii*
BPC422	(RS)-norcoclaurine 6-O-methyltransferase-like	325	7.60E-25	*Jatropha curcas*

Functional classification and identification of differentially expressed genes in library

Annotation of putative transcripts obtained through Blast 2GO analysis showed that many genes are induced in black pepper roots upon colonization by BP25. Majority of the ESTs represented coding sequences present in genomes of *Nelumbo nucifera, Populus trichocarpa, Camellia sinensis, Cicer arietinum* and *Brassica rapa* etc. Analysis of the EST set by the Blast2GO software allowed the annotation of expressed sequences according to the terms of the three main GO vocabularies, i.e. 'biological process', 'molecular function' and 'cellular component'. Functional classification of ESTs showed that many defence related genes are upregulated in roots upon BP25 colonization.

Gene ontology data showed that in a biological process 33%, 30% in cellular component and 51% in molecular process is getting upregulated. The most represented classes of ESTs in terms of 'biological processes' are metabolic process, cellular process response to stimulus and signalling. GO terms included under the category 'response to stimulus' were major pollen allergen Bet v 1-D H-like protein and PR-1, and also 26S protease regulatory subunit, PR-4, catalase, dnaJ homolog and superoxide dismutase induced in response to stress. Major pollen allergen Bet v 1-D H-like protein and PR-1 protein were also categorized under signalling and biological regulation. Less represented category under biological process, cellular component organization included 26S protease regulatory subunit 7-like, actin-depolymerizing factor 7-like protein and 60S ribosomal L5. GO terms such as 5-enolpyruvylshikimate-3-phosphate synthase, probable inositol oxygenase, aquaporin PIP1-3 and 60S ribosomal L5 are involved in cellular amino acid metabolic process, carbohydrate metabolic process, transport and translation respectively. Different categories such as binding, catalytic activity and molecular function regulator could be identified under the main GO vocabulary term 'molecular function'.

InterProScan analysis was done to find out functional motifs in the transcripts and the most represented families identified were Bet v 1 allergen and metallothionein followed by catalase, heat shock protein etc. Enzyme code mapping was done to find the upregulation of enzymes representing different classes, oxidoreductases, transferases and lyases. The KEGG database was queried to assign sequences to specific metabolic and/or biosynthetic pathways. KEGG maps analysis showed that proteins related to different pathways were upregulated. ESTs such as glutathione S-transferase, catalase and inositol oxygenase were found to be involved in different metabolic pathways. Many of the transcripts upregulated are found associated with defense related responses.

Validation of selected transcripts by quantitative Real-time PCR

The expression pattern of transcripts such as PR-4, WRKY transcription factor 40, matallothionein, catalase, glutathione S-transferase was analyzed by qPCR and all the transcripts showed a general increase in the expression in roots bacterized with *P. putida* BP25 (Fig. 7).

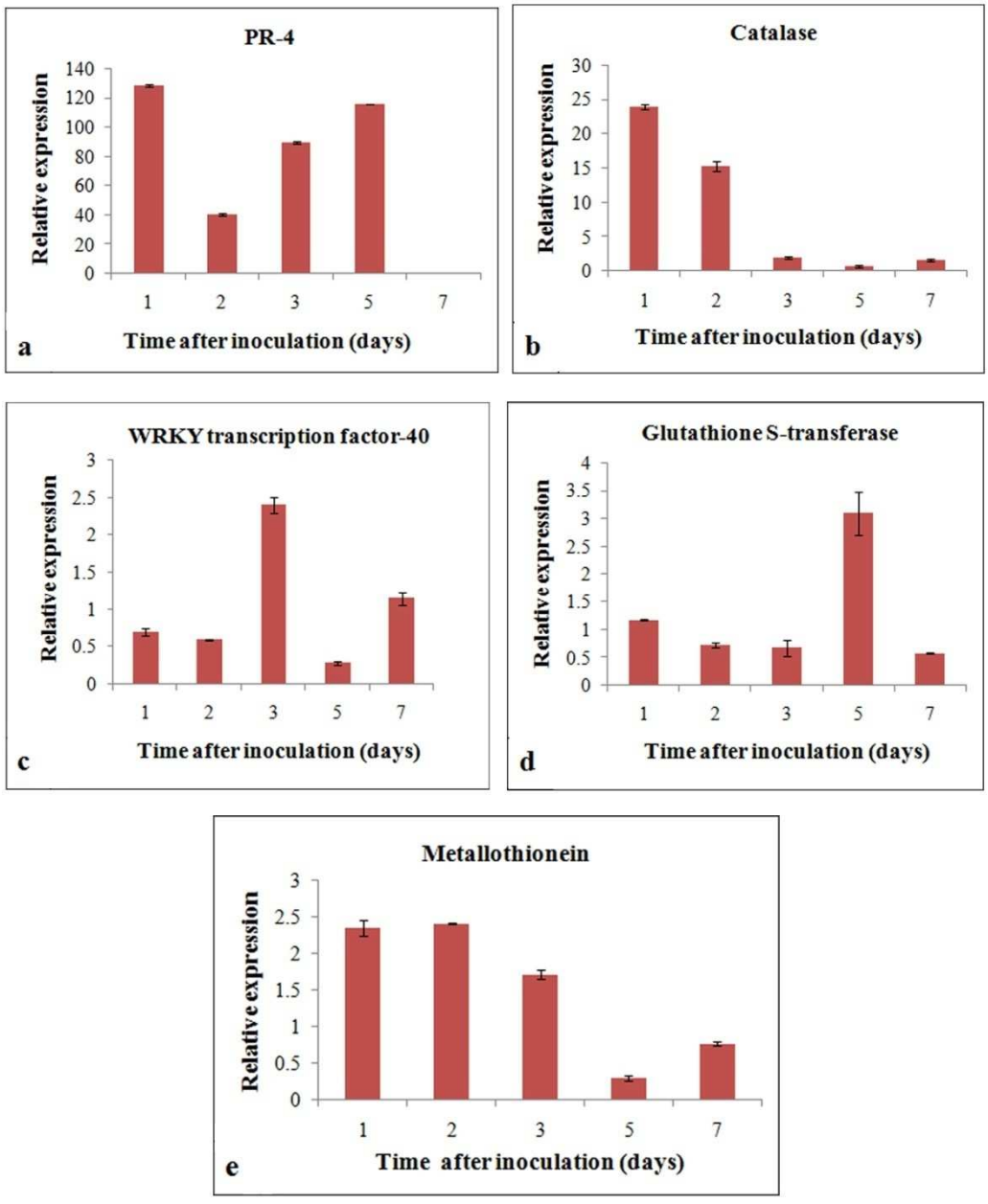

Fig. 7 Relative expression (RE) of transcripts using quantitative RT-PCR. The transcript levels of the genes; PR-4 (a), catalase (b), WRKY transcription factor 40 (c), glutathione S-transferase (d) and metallothionein (e) in black pepper roots colonized with *Pseudomonas putida* BP25 at 1, 2, 3, 5 and 7 dpi. The error bars indicate the standard error of mean values. The C.D. ($p < 0.05$) values are 2.45, 1.36, 0.25, 0.68 and 0.21 for PR-4, catalase, WRKY transcription factor 40, glutathione S-transferase and metallothionein, respectively.

6. CONCLUSIONS

The endophytic nature of *P. putida* BP25 and its colonization pattern in black pepper were clearly proved by this study. The strain having rifampicin resistance aided in tracking and estimating the bacterial population precisely. A population size of 5-6 $\log_{10}$ CFU/g of rhizoplane and up to 4 $\log_{10}$ CFU/g of root could be estimated. A highly sensitive Bio-PCR could prove the presence of the bacteria in lower numbers in the stem of black pepper. Use of a gfp (green fluorescent protein) tagged strain enabled the visualization of bacterial colonization in black pepper. An accurate and rapid real time PCR method was also developed for quantifying *P. putida* BP25R in black pepper tissues. This finding on the endophytic behaviour of the bacterium would be an important biocontrol trait for sustained protection against a range of plant pathogens.

P. putida BP25R was found to produce potential volatile organic compounds (MVOCs) having a significant role in the biocontrol potential of this bacterium. MVOCs such as 2,5-dimethyl pyrazine, methyl pyrazine, dimethyl trisulfide, 2-ethyl 5-methyl pyrazine and 2-ethyl 3,6-dimethyl pyrazine displayed inhibitory activity against a broad spectrum of pathogens such as *Phytophthora capsici*, *Rhizoctonia solani*, *Colletotrichum gloeosporioides*, *Athelia rolfsii*, *Gibberella moniliformis* and *Radopholus similis*. Among the compounds, dimethyl trisulfide exhibited 100% inhibition even at a low concentration of 2.65µg/cm^3 against fungal and oomycetal pathogens and, a plant parasitic nematode, *R. similis*. *In planta* assays were done on black pepper cut shoots against *P. capsici* and shown to have significant inhibitory activity. Pyrazine compounds efficiently caused 100% lesion suppression on black pepper cut shoots at a concentration of21 µg/cm^3 and dimethyl trisulfide was found effective at lower concentrations. It was also found that dimethyl trisulfide can be exploited as a soil fumigant against soilborne pathogens. So, the inhibiting potential of MVOCs might provide an alternative strategy for eco-friendly pest management in agriculture.

The interaction between *P. putida* BP25 and black pepper is found associated with induction of resistance in the host plant. In the present study, biochemical analyses were done and increase in phenol content, lignin and, defense related enzymes such as peroxidase, catalase and phenylalanine ammonia lyase were observed. The molecular responses were studied by constructing a cDNA library using a PCR based suppression subtractive hybridization (SSH) method which enabled the identification of differentially expressed transcripts. Many defence related transcripts such as pathogenesis related proteins, catalase, glutathione S-transferase, 5-enolpyruvylshikimate-3-phosphate synthase etc. were found

associated with resistance induction. The upregulation of selected transcripts were validated by quantitative real time PCR (qPCR) method. This differential expression of transcripts associated with effective activation of plant immune responses may confer broad spectrum resistance to plant pathogens.

It can be concluded that the strain, *P. putida* BP25 possess several beneficial biocontrol traits which helps in potentiating the plant health. Hence, it can be exploited as a microbial formulation for sustainable crop protection.

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8. PUBLICATIONS FROM THESIS

Agisha VN, Eapen SJ, Bhai RS and Kumar A (2017) Detecting and monitoring endophytic colonization by *Pseudomonas putida* BP25 in black pepper (*Piper nigrum* L.) using quantitative real-time PCR. *Journal of Spices and Aromatic Crops*, 26(1). (Accepted for publication).

Neelam S, **Agisha V N**, Vibhuti M, Aditi K, Subaharan K, Vibina V, R Suseelabhai , SJ Eapen and A Kumar (2015) Genetic analysis of plant endophytic *Pseudomonas putida* BP25 and chemo-profiling of its antimicrobial volatile organic compounds. *Microbiological research* 173: 66-78.

VN Agisha, S Neelam, M Vibhuti, SJ Eapen, R Suseelabhai and A Kumar (2016) Microbial volatile organic compounds (MVOCs) from endophytic bacterium *Pseudomonas putida* BP25R inhibit a broad spectrum of plant pathogens. In *abstracts: 6th International conference, Indian Phytopathological Society: Plant, Pathogens and People*. ISBN: 81-7019-527-2. p.118, February 23-27, New Delhi, India.

Agisha VN, Neelam S, Vibhuti M, Santhosh J Eapen and A Kumar (2014) Tracing endophytic colonization of black pepper (*Piper nigrum* L.) by *Pseudomonas putida* BP25, a broad spectrum biocontrol agent against oomycete and fungal pathogens. In *abstracts: International symposium on plantation crops (PLACROSYM XXI)* ISBN: 978-81-86872-48-2. p.166, December 10-12, Kozhikode, Kerala, India.

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